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Cloning, Heterologous Expression, and Characterization of the Gene Cluster Required for Gougerotin Biosynthesis

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SUMMARY

Gougerotin, a peptidyl nucleoside antibiotic, possesses antitumor, antiviral, antibacterial, antimycoplasma, anthelmintic, and acaricidal activities. Here, we report the cloning of a complete gougerotin biosynthetic gene cluster from *Streptomyces graminearus* and heterologous production of gougerotin in *Streptomyces coelicolor*. Sequence analysis of a 28.7 kb DNA fragment indicated that the cluster consists of 25 open reading frames (ORFs). Gene disruption and genetic complementation experiments revealed that 15 of the 25 ORFs are required for gougerotin biosynthesis. A gougerotin biosynthetic pathway was proposed based on the analyses of bioinformatics and intermediates accumulated in selected gene inactivation mutants. These studies substantially promoted our understanding of gougerotin biosynthesis and provide "building blocks" for combinatorial biosynthesis using genes encoding different enzymes in nucleoside antibiotics.

INTRODUCTION

The peptidyl nucleoside antibiotics are a group of important microbial secondary metabolites with a broad spectrum of biological activities. Gougerotin, also known as aspiculamycin or asteromycin, is a peptidyl nucleoside antibiotic produced by *Streptomyces gougerotii* (Iwasaki, 1962), *Streptomyces toyocaensis* var. *aspiculamyceticus* (Arai et al., 1974), and *Streptomyces* S-514 (Ikeuchi et al., 1972). Gougerotin has been found to be a specific inhibitor of protein synthesis (Clark and Gunther, 1963). It has been shown to inhibit selectively the incorporation of amino acids into protein by binding ribosomal peptidyl transferase (Casjens and Morris, 1965; Cerná et al., 1971). The interaction of gougerotin with peptidyl transferase disturbs the relative positioning of the 3′ end of the P/P′ site-bound tRNA and the peptidyl transferase loop region of 23S rRNA (Kirillov et al., 1999). This mode of action affords gougerotin versatile bioactive properties, and it is reported to possess antibacterial, anthelmintic, acaricidal, antimycoplasma, antiviral, and antitumor activities (Haneishi et al., 1974; Kondo et al., 1974; Lacal et al., 1980).

The peptidyl nucleoside antibiotics are generated from simple nucleoside and amino acid building blocks. Most members of this family of antibiotics are consisted of three distinct structural components: a heterocyclic base, an amino sugar appendage, and an unusual amino acid or peptidyl moiety (Cone et al., 2003). Gougerotin consists of cytosine, 4-amino-4-deoxyglucuronamide, and a sarcosyl-D-serine dipeptide (Figure 1A). Cytosine and 4-amino-4-deoxyglucuronamide constitute the nucleoside skeleton (Fox et al., 1968), which resembles cytosylglucuronic acid (CGA) intermediate in blasticidin S biosynthesis (Cone et al., 2003). Gougerotin is a stereoisomer of ningnanmycin, a plant virucide commercially available in China (Wang et al., 2010). The only difference between these two antibiotics is that ningnanmycin contains L-serine, and gougerotin contains D-serine in the dipeptide moiety (Figure 1A).

Biosynthetic pathways for some of peptidyl nucleoside antibiotics have been extensively studied in the past decades. The biosynthetic gene clusters were identified for the adenine derivative puromycin (Lacalle et al., 1992; Tercero et al., 1996), the cytosine derivative blasticidin S (Cone et al., 2003), mildiomycin (Li et al., 2008), as well as uridine-based nikkomycin and polyoxin (Chen et al., 2009; Liu et al., 2005). Previous studies of gougerotin have focused on its mechanism of action, biological activities, and chemical synthesis (Clark and Gunther, 1963; Fox and Watanabe, 1971; Haneishi et al., 1974; Kirillov et al., 1999; Kondo et al., 1974; Lacal et al., 1980; Lichtenthaler et al., 1971; Migawa et al., 2005; Watanabe et al., 1972). However, to our knowledge, nothing is known about the gougerotin biosynthesis. To understand the biosynthesis of gougerotin, we cloned the complete gougerotin biosynthetic gene cluster from *S. graminearus*, a gougerotin-producing strain deposited as CGMCC 4.506 at China General Microbiological Culture Collection (CGMCC). The heterologous production of gougerotin in the well-characterized surrogate *S. coelicolor* helped us to determine the border of gene cluster for gougerotin biosynthesis. Bioinformatics and functional analyses of open reading frames (ORFs) in the gene cluster led to a proposed gougerotin biosynthetic pathway. This study provides significant insight into the biosynthesis of gougerotin. It will also pave the way for the generation of hybrid peptidyl nucleoside antibiotics as exemplified by two recent studies (Li et al., 2011; Zhai et al., 2012).

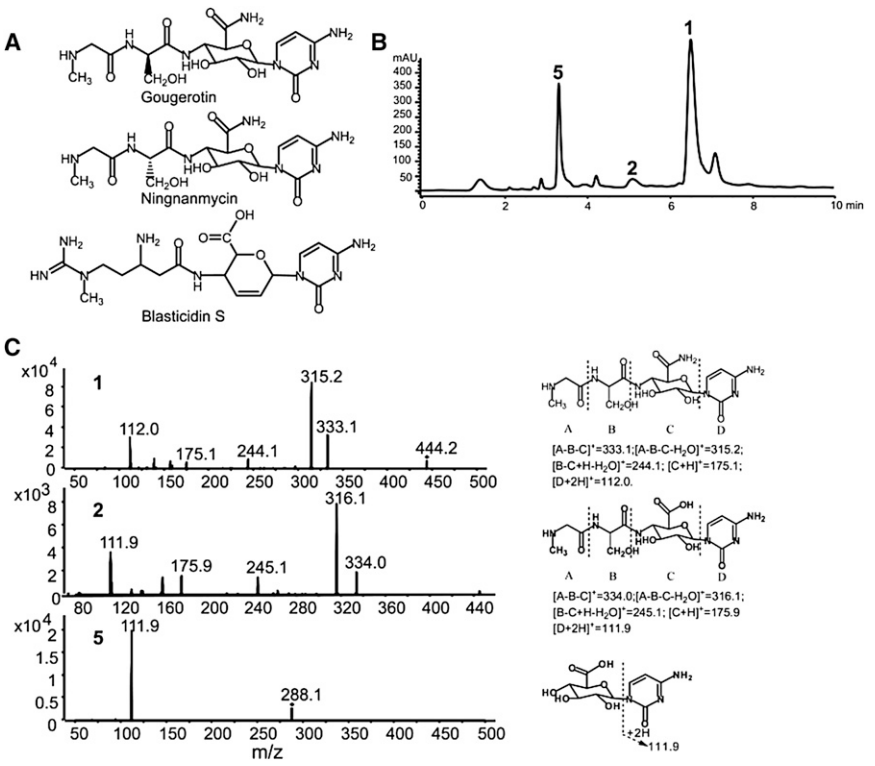

Figure 1. Identification of Gougerotin from *S. graminearus*

(A) Chemical structures of gougerotin, ningnanmycin, and blasticidin S.

(B) HPLC analysis of the metabolites produced by *S. graminearus*.

(C) MS/MS analysis of the metabolites produced by *S. graminearus*.

1: gougerotin, $[M+H]^+$ = 444.2 (exact mass = 443.18); **2**: yunnanmycin, $[M+H]^+$ = 445.2 (exact mass = 444.16); **5**: CGA, $[M+H]^+$ = 288.1 (exact mass = 287.08). The fragmentation patterns of the parent ions are shown, and the peaks corresponding to the characteristic fragments are labeled.

See also Figure S1.

RESULTS

Identification of Gougerotin from *S. graminearus*

Gougerotin was previously isolated from several *Streptomyces* by Arai et al. (1974), Ikeuchi et al. (1972) and Iwasaki (1962). In this study, culture filtrate of *S. graminearus*, a gougerotin producer deposited as CGMCC 4.506 at CGMCC, was examined by HPLC analysis. Three distinct peaks were identified (Figure 1B). Isolated compound **1** was first analyzed by electrospray ionization mass spectrometry (ESI-MS). ESI-MS analysis of **1** revealed an $[M+H]^+$ ion at m/z 444.2 (exact mass = 443.4), and the MS/MS spectrum revealed the fragmentation pattern consistent with that for gougerotin (Figure 1C). The structure of **1** was finally validated by NMR analysis (Figure S1 available online). From the NMR spectra, we noticed two sets of signals for 16-CH, 19-CH, 21-CH, 13-NH, and 17-NH (Figure S1). We speculate the coexistence of its stereoisomer (ningnanmycin) with gougerotin. In order to test this possibility, purified compound **1** was subjected to acid hydrolysis and following derivatization with o-phthaldialdehyde (OPA) and *N*-acetyl-L-cysteine (NAC) (Figures S3A and S3B). The presence of both D-serine and L-serine was confirmed after HPLC separation of D- and L-serine derivatives (Figure S3C). Ningnanmycin may be generated as an intermediate of gougerotin biosynthesis or from the racemization of gougerotin during the sample handling process. In addition, we also detected CGA (**5**) (Figures 1B, 1C, and S2; Table S1) and a minor metabolite yunnanmycin (**2**) (Figures 1B and 1C) (Chen et al., 1998), which may be from the accumulated intermediate of gougerotin production due to incomplete biotransformation.

Cloning and Characterization of the Gougerotin Gene Cluster

Based on the observation that gougerotin and blasticidin S share similar nucleoside skeleton (Figure 1A), we speculated that 4-amino-glucuronamide nucleoside skeleton of gougerotin is a derivative of CGA. In blasticidin S biosynthesis, CGA synthase (encoded by *blsD*) catalyzes the condensation of cytosine and UDP-glucuronic acid to form CGA (Gould and Guo, 1994). By searching the genome sequence of *S. graminearus* generated from 454 sequencing (unpublished data), we identified a BlsD homolog (45% similarity, 34% identity). This gene was then used as a probe to clone the entire gougerotin biosynthetic gene cluster from a fosmid library consisting of ~8,000 clones from *S. graminearus* genomic DNA. PCR screening of the library with the *blsD* homologous gene primers (gouF-F and gouF-R) revealed 5 positive clones from ~2,000 fosmid clones. Among them, fosmid D6-4H and D6-9F are overlapping and span ~40 kb contiguous region as revealed by end-sequencing and alignment with the genome sequence of *S. graminearus*. Further analysis of fosmid D6-4H shows that it contains a 28.7 kb insert (GenBank accession number JQ307220) with an overall G+C content of 72.70%. The FramePlot 4.0beta online program revealed the organization of the cluster consisting of 25 complete ORFs (Figure 2), and the deduced functions of genes in this cluster are listed in Table 1.

To test whether the 28.7 kb contiguous DNA fragment is responsible for gougerotin biosynthesis, we set out to inactivate the *gouH* and *gouL* by targeted double-crossover recombination in *S. graminearus*. Culture filtrate from the resulting *gouH* and *gouL* inactivation mutants was subjected to HPLC analysis. Unlike culture filtrate from *S. graminearus* wild-type strain, which shows a distinct gougerotin peak with a retention time at ~6.4 min, culture filtrates from *gouH* and *gouL* inactivation mutants lack this peak (Figure 3). Because *gouH* and *gouL* are within a group of 12 genes that might form an operon, inactivation of downstream genes by polar effects on transcription might have contributed to the phenotype observed. To exclude this possibility, we selected the upstream *gouL* gene for genetic complementation experiments. Complementation

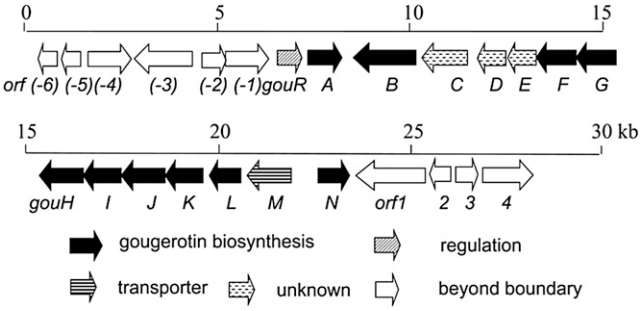

Figure 2. Organization of the Gougerotin Biosynthetic Gene Cluster in Fosmid D6-4H

The gene cluster spans 28.7 kb fragment and contains 25 ORFs. Genes are indicated by different arrowheads according to their proposed roles.

of *gouL* mutant with a functional copy of *gouL* under the control of constitutive *hrdB* promoter restored the production of gougerotin (Figure 3). These results show that the gene cluster we cloned is indeed involved in gougerotin biosynthesis.

Heterologous Production of Gougerotin in *S. coelicolor*

To test whether D6-4H contains all genes required for gougerotin biosynthesis, the insert of 28.7 kb was cloned into pSET152, and the resulting recombinant plasmid pSET152::D6-4H (Figure S4A) was first introduced into *S. coelicolor* M145 to generate M145-D6-4H. Gougerotin was detected in the culture filtrate of M145-D6-4H. However, the peak of gougerotin was interfered by surrounding peaks from the host (data not shown). We then chose the engineered *S. coelicolor* M1146 as a heterologous host in which four endogenous secondary metabolite gene clusters were deleted (Gomez-Escribano and Bibb, 2011). Culture filtrate from M1146-D6-4H was subjected to HPLC analysis, and a distinct peak with a retention time of ~6.4 min appeared, whereas the culture filtrates from the negative control strains (*S. coelicolor* M1146 and *S. coelicolor* M1146-pSET152) lack this peak (Figure 4A). To further confirm that the peak from the recombinant strain was indeed gougerotin, we performed the MS/MS analysis in a positive ion mode, which revealed a distinct ion and fragmentation pattern that matches exactly to the pattern of the peak from *S. graminearus* wild-type (Figure 4B). These results definitively demonstrated that we have successfully cloned the complete gene cluster for gougerotin biosynthesis, which is sufficient for heterologous production in the surrogate host.

Boundaries of the Gougerotin Biosynthetic Gene Cluster

In order to determine the minimal contiguous gene cluster required for gougerotin production, deletion derivatives that truncated the fosmid D6-4H from both sides of the 28.7 kb insert were constructed (Figures S4B and S4C) and transferred into *S. coelicolor* M1146. The effects of these deletions on gougerotin production were monitored by HPLC analysis. On the left-hand side, a recombinant strain, BDorf(−1)–(−6) lacking ORFs(−6)–(−1), still produced gougerotin (Figure 4C). *gouR*, encoding a putative TetR family regulatory protein, is the only gene with potential regulatory function in the cluster. A separate study shows that *gouR* plays a regulatory role in gougerotin biosynthesis (unpublished data). Thus, we propose that *gouR* is at the left boundary of the gougerotin biosynthetic gene cluster. On the right-hand side, the strain, BDorf1–4 with the deletions of ORFs1–4, continued to produce gougerotin, whereas another strain, BDgouN, in which *gouN* was removed in addition to ORFs1–4, abolished gougerotin production (Figure 4C). These experiments suggest that the minimum gougerotin biosynthetic gene cluster contains 15 genes, from *gouR* to *gouN*, spanning a contiguous DNA fragment of ~17 kb. This DNA fragment is sufficient to confer gougerotin production in *S. coelicolor* M1146. Bioinformatics analysis, consisting of BLAST searches followed by alignment to representative proteins and identification of conserved active site residues, allowed us to assign potential functions to each ORF (Table 1).

Biosynthesis of Nucleoside Skeleton

Four genes, *gouA*, *gouB*, *gouF*, and *gouH*, are putatively involved in the biosynthesis of the nucleoside moiety of gougerotin. As mentioned above, gougerotin and blasticidin S share similar nucleoside skeleton. In blasticidin S biosynthesis, CGA is the earliest committed intermediate. CGA synthase (encoded by *blsD*) catalyzes the condensation of UDP-glucuronic acid and cytosine (Gould and Guo, 1994). The presence of CGA in the culture supernatant of *S. graminearus* (Figures 1B and 1C) indicated that CGA is also an intermediate in gougerotin biosynthesis. By blasting with the GenBank database, GouF is found to exhibit 34% identity with the BlsD from the biosynthetic pathway of blasticidin S (Cone et al., 2003). It also exhibits 28% identity with the AmiJ, which is proposed to catalyze the coupling of amicetose and cytosine to form an N-glycosidic bond in amicetin biosynthesis (Zhang et al., 2012). Thus, GouF possibly catalyzes the coupling of UDP-glucuronic acid and cytosine to form CGA in gougerotin biosynthesis. To test this hypothesis in vivo, *gouF* mutant cluster was constructed and then introduced into *S. coelicolor* M1146 to generate gouF-IFD, CGA formation and gougerotin production were completely abolished, and cytosine was accumulated substantially in the gouF-IFD strain, which is verified by the coinjection of supernatant from gouF-IFD strain with cytosine standard (Figure 5A). GouA exhibits high identity to various putative oxidoreductases, such as the putative oxidoreductase (CAJ89749.1, 88% identity) from *Streptomyces ambofaciens* ATCC 23877. GouA has the D-arabinose dehydrogenase-like domain and potential NAD-binding sites, substrate-binding sites, and Zn-binding sites. Therefore, GouA may oxidize CGA at C-4 of glucuronic acid, and the resulting 4-carbonyl-CGA could serve as a substrate for amination catalyzed by GouH to form 4-amino-CGA. GouH, a DegT/DnrJ/EryC1/StrS aminotransferase with pyridoxal-phosphate as the coenzyme, displays 33% (122 of 372) identity to BlsH, which is predicted to function in the amino deoxynucleoside formation in the biosynthesis of blasticidin S (Cone et al., 2003). In the resulting *gouH* inactivation mutant of *S. graminearus*, gougerotin was not produced, and CGA instead of its predicted substrate of 4-carbonyl-CGA was accumulated (Figure 3). The same result was observed with the *gouH* in-frame deletion mutant in *S. coelicolor* M1146 (Figure 5A). This can be explained by the unstable property of 4-carbonyl-CGA. The asparagine synthase (glutamine hydrolyzing) encoded by *gouB* may be responsible for the amidation of yunnanmycin at C-6 of glucuronic acid (Figure 6).

Table 1. Summary of Fosmid D6-4H ORFs

ORF	No. of Amino Acids	Proposed Function	Protein Homology[a]	Amino Acid Identity (%)
orf(−6)	117		*Streptomyces zinciresistens* K42 hypothetical protein SZN_16877 (ZP_08804424)	42/114 (37)
orf(−5)	132		*Streptomyces pristinaespiralis* ATCC 25486 predicted protein (ZP_06913373)	88/125 (70)
orf(−4)	307		*Streptomyces cattleya* NRRL 8057 = DSM 46488 phosphatidylserine/phosphatidylglycerophosphate/cardiolipin synthase-like protein (AEW98033)	164/280 (59)
orf(−3)	500		*Streptosporangium roseum* DSM 43021 major facilitator superfamily MFS_1 (ACZ86657)	408/500 (82)
orf(−2)	123		*Streptomyces cattleya* NRRL 8057 = DSM 46488 YCII-related protein (AEW99749)	104/123 (85)
orf(−1)	403		*Frankia sp.* EUN1f putative RNA polymerase, sigma-24 subunit, ECF subfamily (ZP_06415013)	342/402 (85)
gouR	228	Transcriptional regulator	*Streptomyces ambofaciens* ATCC 23877 putative TetR-family transcriptional regulator (CAJ89747)	153/204 (75)
gouA	346	CGA oxidase	*Streptomyces ambofaciens* ATCC 23877 putative oxidoreductase (CAJ89749)	303/346 (88)
gouB	595	Amidase	*Cytophaga hutchinsonii* ATCC 33406 asparagine synthase (ABG60840)	161/552 (29)
gouC	433	Unknown	*Streptomyces sp*. SPB74 conserved hypothetical protein (EDY43555)	129/467 (28)
gouD	315	Unknown	*Streptomyces ghanaensis* ATCC 14672 glycosyltransferase (EFE71578)	128/290 (44)
gouE	246	Unknown	*Planctomyces brasiliensis DSM* 5305 Conserved hypothetical protein CHP00730 (ADY58906)	73/182 (40)
gouF	397	CGA synthase	*Streptomyces griseochromogenes* CGA synthase (AAP03118)	75/222 (34)
gouG	388	Phosphoglycerate mutase	*Moorella thermoacetica* ATCC 39073 phosphoglycerate mutase (ABC19419)	62/193 (32)
gouH	386	Aminotransferase	*Methanospirillum hungatei* JF-1 DegT/DnrJ/EryC1/StrS aminotransferase (ABD41833)	102/344 (30)
gouI	311	Aminotransferase	*Synechococcus sp*. WH 5701 perosamine synthetase (ZP_01083876)	64/142 (45)
gouJ	372	Acyl-CoA N-acyltransferase	*Bradyrhizobium sp*. ORS 278 putative Acyl-CoA N-acyltransferase (CAL78697)	50/164 (30)
gouK	224	Acyl-CoA synthetase	*Clostridium botulinum* A3 str. Loch Maree CoA-binding domain protein (ACA54930)	45/227 (20)
gouL	269	2-Aminomalonate semialdehyde oxidase	*Pseudomonas syringae pv*. syringae FF5 3-hydroxyisobutyrate dehydrogenase (ZP_06495709)	77/218 (35)
gouM	442	Transporter	*Anabaena variabilis* ATCC 29413 major facilitator superfamily MFS_1 (ABA25090)	49/157 (31)
gouN	239	Glycine N-methyltransferase	*Microcoleus chthonoplastes* PCC 7420 Methyltransferase domain family (EDX77140)	58/187 (31)
orf1	734		*Streptomyces roseosporus* NRRL 11379 peroxidase/catalase (EFE72825)	639/733 (87)
orf2	130		*Aspergillus clavatus* NRRL 1 dynactin, putative (EAW14193)	30/89 (34)
orf3	138		*Streptomyces sp*. SirexAA-E ferric uptake regulator, Fur family (AEN12271)	119/136 (88)
orf4	380		*Streptomyces clavuligerus* ATCC 27064 truncated integral membrane protein (EDY49466)	196/346 (57)

The percentage of homology presented is based on overall homology.
[a]Results generated by BLAST analysis of deduced ORFs are presented. Accession numbers are listed in parentheses.

Biosynthesis and Incorporation of Peptidyl Moiety

The peptidyl moiety of gougerotin consists of a sarcosyl-D-serine dipeptide that most likely comes from serine and sarcosine. Three genes (*gouG*, *gouI*, and *gouL*) are predicted to participate in the de novo biosynthesis of serine. A putative phosphoglycerate mutase encoded by *gouG* is responsible for the conversion of 3-phosphoglycerate (3PG) to 2-phosphoglycerate (2PG) through a 2,3-bisphosphoglycerate intermediate. GouI shows 45% identity to a perosamine synthetase (ZP_01083876.1) from *Synechococcus sp.* WH 5701 and may be

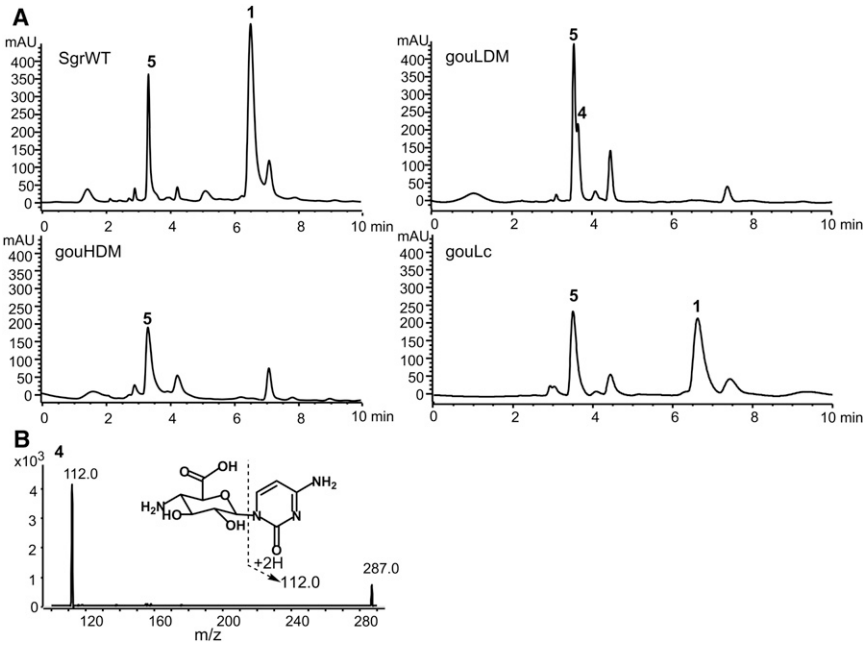

Figure 3. HPLC Analysis of Gougerotin Produced by *S. graminearus* and Its Derivatives

(A) HPLC analysis of gougerotin production in *gouH* mutant, *gouL* mutant, and *gouL* complementation strain. SgrWT, supernatant collected from *S. graminearus* wild-type; gouHDM, supernatant collected from *S. graminearus gouH* inactivation mutant; gouLDM, supernatant collected from *S. graminearus gouL* inactivation mutant; gouLc, supernatant collected from *S. graminearus gouL* complementation strains. **1**, gougerotin; **4**, 4-amino CGA; **5**, CGA.

(B) MS/MS analysis of the intermediate **4** accumulated in the *gouL* mutant.

See also Figure S2.

involved in the formation of 2-aminomalonate semialdehyde, which can putatively be oxidized to serine by GouL. GouL is homologous to many 3-hydroxyisobutyrate dehydrogenases, which could catalyze not only the oxidation of 3-hydroxyisobutyrate but also L-serine, D-threonine, and other 3-hydroxyacid derivates (Yao et al., 2010). Inactivation of *gouL* resulted in the accumulation of compound **4** with $[M+H]^+$ = 287.1 (exact mass = 286.09) (Figure 3). MS/MS analysis showed that this compound is 4-amino-CGA, lacking the dipepetide compared with yunnanmycin (Figure 3). Sarcosine (*N*-methyl-glycine) is generated from glycine by glycine N-methyltransferase (Velichkova and Himo, 2005). In actinomycin biosynthesis, glycine was first methylated to form sarcosine, which is then incorporated into the actinomycin peptide backbone (Fukagawa et al., 1974). GouN carries a methyltransferase domain and is probably involved in the methylation of glycine to form sarcosine. This is verified by the accumulation of a compound **3** with $[M+H]^+$ = 374.1 (exact mass = 373.12), lacking sarcosine compared with yunnanmycin (**2**) in the BDgouN strain (Figures 4C and 5B). We also tried feeding different concentrations of D- and L-serine into *gouL* mutant culture or sarcosine into *gouN* mutant culture, but no restoration of gougerotin production was observed.

When serine and sarcosine are ready, they need to be incorporated into the nucleoside skeleton to form an active gougerotin. There are two possible ways for the incorporation of serine and sarcosine. First, serine and sarcosine residues can be sequentially attached to the nucleoside skeleton. Second, serine and sarcosine are condensed to form the sarcosyl-serine dipeptide, which is then attached to the nucleoside skeleton. The accumulation of compound **3** in the *gouN* mutant indicated that the nucleoside moiety is coupled with serine at first and then sarcosine is attached to the seryl-nucleoside moiety skeleton.

The incorporation step may require the participation of *gouJ* and *gouK*. GouK shows 20% identity and 37% similarity to coenzyme A (CoA)-binding domain-containing protein (YP_001788025.1) from *Clostridium botulinum* (strain Loch Maree/Type A3). Although the N terminus of GouJ contains a conserved NAT_SF domain for *N*-acetyltransferase superfamily enzymes and displays homologous to acyl-CoA *N*-acyltransferase (YP_001206914.1, 30% identity, 41% similarity) from *Bradyrhizobium sp.* ORS 278, *N*-acetyltransferases can transfer the acetyl group from acetyl CoA to a primary amine (Dyda et al., 2000). Therefore, we speculate that GouK can activate the serine or glycine to form seryl-CoA or sarcosyl-CoA, and GouJ can then catalyze the transfer of the seryl and sarcosyl group from acyl-CoA to the amino group of nucleoside moiety. In *gouK* mutant (gouK-IFD), gougerotin was not produced, and we found a peak of compound **4** with $[M+H]^+$ = 287.1 (exact mass = 286.09) (Figure 5A). MS/MS analysis showed that this compound is 4-amino-CGA (Figure 5C). This is consistent with the involvement of GouK in the condensation of nucleoside moiety and peptidyl group.

Regulation and Secretion

One candidate regulatory gene, *gouR*, was identified. It encodes a putative TetR family transcriptional regulator that is characterized by the N-terminal helix-turn-helix (HTH) DNA-binding domain. TetR is a large family of transcriptional regulators, which often serve as repressors and are widely distributed among bacteria, regulating a number of diverse processes (Ramos et al., 2005; Routh et al., 2009). The detailed functions of this gene in gougerotin biosynthesis will be addressed in a separate study.

Functioning as a specific inhibitor of protein synthesis, gougerotin may be toxic to its producer. A simple way to avoid this detrimental effect is the secretion of this compound outside of the cell. The *gouM* gene encodes a protein homologous to permease of the major facilitator superfamily (MFS). MFS transporters comprise a large and diverse group of membrane proteins that facilitate the transport of a variety of substrates across cytoplasm or internal membranes (Law et al., 2008). The MFS transporters are single polypeptide carriers that are only capable of transporting small molecules by using energy from electrochemical gradients across membranes. This is different from the most common ATP-binding cassette (ABC) transporters, which are generally multicomponent proteins capable of

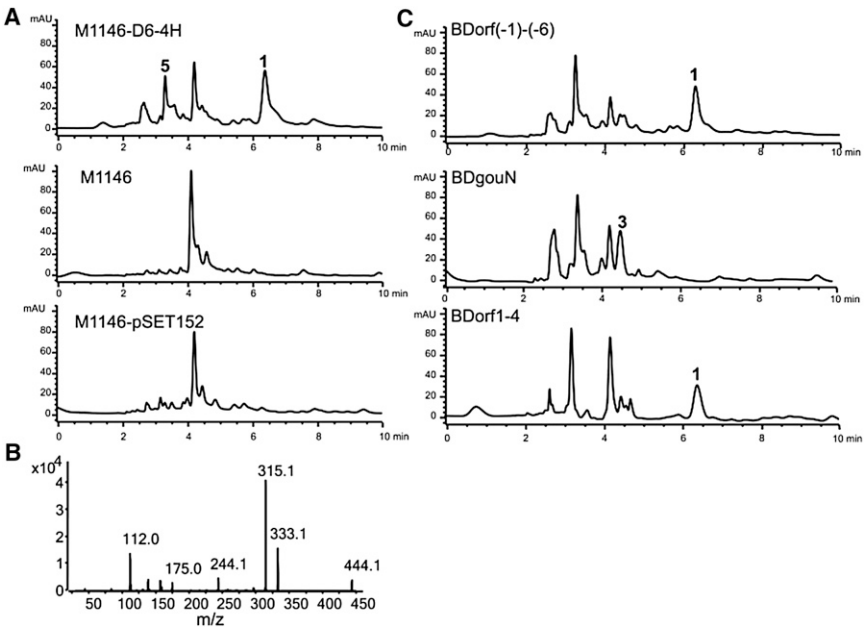

Figure 4. Heterologous Expression of Gougerotin and Boundary Determination

(A) Heterologous expression of gougerotin in *S. coelicolor* M1146.

(B) MS/MS analysis of gougerotin produced by *S. coelicolor* M1146-D6-4H.

(C) HPLC analysis of gougerotin produced by mutants for boundary determination. M1146-D6-4H, supernatant collected from *S. coelicolor* M1146 containing pSET152::D6-4H; M1146, supernatant collected from *S. coelicolor* M1146; M1146-pSET152, supernatant collected from *S. coelicolor* M1146 containing pSET152; BDorf(−1)–(−6), supernatant collected from mutant lacking ORFs(−6)–(−1); BDgouN supernatant collected from mutant lacking *gouN* and ORFs1–4; BDorf1–4, supernatant collected from mutant lacking ORFs1–4. **1**, gougerotin; **3**, compound **3**; **5**, CGA.

See also Figure S4.

transporting both small molecules and macromolecules at the expense of ATP hydrolysis (Martín et al., 2005). Like GriC from griseobactin cluster (Patzer and Braun, 2010) and HomH from holomycin cluster (Huang et al., 2011), GouM can potentially export gougerotin from the cytoplasm into the medium.

Genes with Unknown Functions

Finally, the functions of three genes are not assigned. *gouD* encodes a glycosyltransferase and shows 44% identity to ZP_06581117.1 from *Streptomyces ghanaensis* ATCC 14672, 42% identity to NP_822010.1 from *Streptomyces avermitilis* MA-4680. The deduced products of *gouC* and *gouE* exhibit sequence similarity to hypothetical protein (ZP_06827477.1, 28% identity) from *Streptomyces sp*. SPB74 and Plabr_1294(YP_004268928.1, 40% identity) from *Planctomyces brasiliensis* DSM 5305, respectively. More genetic and biochemical studies are needed to clarify the exact functions of these genes.

DISCUSSION

Gougerotin is a peptidyl nucleoside antibiotic with broad biological activities. In this study, the gene cluster for gougerotin biosynthesis was cloned. This is based on the observation that 4-amino-glucuronamide nucleoside skeleton of gougerotin is very similar to the CGA intermediate in blasticidin S biosynthesis. An amide group was present in 4-amino-glucuronamide instead of carboxyl group in CGA. In blasticidin S biosynthesis, CGA synthase encoded by *blsD* is responsible for the condensation of cytosine and UDP-glucuronic acid to form CGA (Gould and Guo, 1994). By searching the sequenced genome of *S. graminearus*, we identified GouF as a homolog of BlsD. We initially cloned a 28.7 kb DNA fragment that covers all genes required for gougerotin biosynthesis. This is demonstrated by the successful production of gougerotin in the heterologous host *S. coelicolor* M1146. The boundary of the gene cluster was determined by generating mutants with truncations from both sides of the 28.7 kb contiguous region. The minimized gene cluster consists of 15 genes: 13 of them encode enzymes responsible for gougerotin biosynthesis, 1 encodes a putative pathway-specific regulatory protein, and the other one encodes a potential gougerotin secretion protein. Bioinformatics analysis and the identification of intermediates accumulated in specific gene inactivation mutants led to a proposed biosynthetic pathway of gougerotin (Figure 6). Cytosine (**6**) is first coupled with UDP-glucuronic acid to form CGA (**5**), then CGA is oxidized and aminated to obtain 4-amino-CGA (**4**). Next, activated serine and sarcosine are sequentially attached to 4-amino-CGA to form yunnanmycin (**2**). Final generation of gougerotin (**1**) was achieved after the amidation of yunnanmycin at C-6 of glucuronic acid.

The first step proposed in the pathway requires UDP-glucuronic acid and cytosine. UDP-glucuronic acid was suggested to originate from the primary precursor glucose in blasticidin S biosynthesis (Cone et al., 2003). Cytosine is proposed to be generated from the hydrolysis of cytidine monophosphate (CMP) by CMP hydrolase. Homologs of the CMP hydrolase were identified in blasticidin S, mildiomycin, and amicetin biosynthetic gene clusters (Cone et al., 2003; Li et al., 2008; Zhang et al., 2012). Constitutive expression of *blsM*, the gene encoding the CMP hydrolase in blasticidin S cluster, in *S. lividans* TK24 led to 20- to 27-fold increase of cytosine levels over the wild-type *S. lividans* TK24 (Cone et al., 2003). The CMP hydrolysis activity of MilB from mildiomycin gene cluster was demonstrated in vitro, and the *milB* disruption mutant abolished mildiomycin production (Li et al., 2008). Likewise, the inactivation mutant of CMP hydrolase gene (*amil*) in *S. vinaceusdrappus* NRRL 2363 lost the ability to produce amicetin, and the complementation strain could partially restore the production of amicetin (Zhang et al., 2012). In the biosynthetic pathway of these three compounds, CMP hydrolase is responsible for the hydrolysis of CMP to provide cytosine for the following coupling reactions. Although a CMP hydrolase-coding gene was located at a locus apart from the gougerotin cluster on the chromosome of *S. graminearus*, the gougerotin biosynthetic gene cluster alone

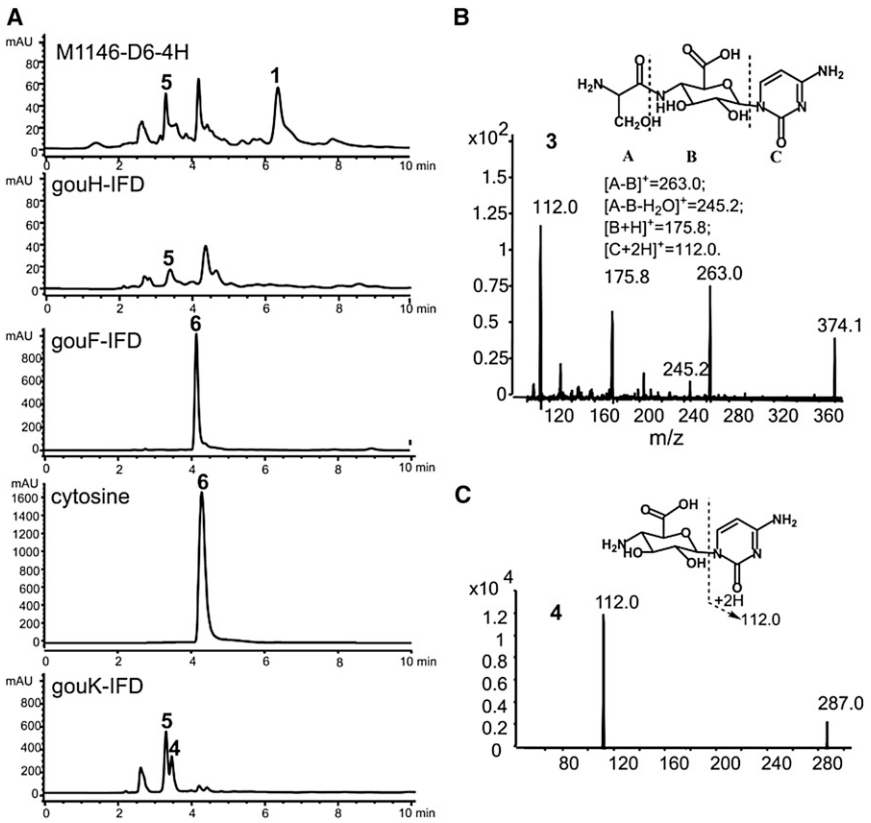

Figure 5. Intermediates Accumulation in Selected Mutants

(A) HPLC analysis of intermediates accumulated in *gouF*, *gouH*, and *gouK* in-frame deletion mutants.

(B) MS/MS analysis of the intermediate **3** accumulated in the BDgouN strain.

(C) MS/MS analysis of the intermediate **4** accumulated in the *gouK* in-frame deletion mutant.

M1146-D6-4H, supernatant collected from *S. coelicolor* M1146 containing pSET152::D6-4H; gouH-IFD, supernatant collected from *gouH* in-frame deletion mutant; gouF-IFD, supernatant collected from *gouF* in-frame deletion mutant; cytosine, cytosine standard (0.25 mg/ml); gouK-IFD, supernatant collected from *gouK* in-frame deletion mutant; **1**, gougerotin; **3**, compound **3**; **4**, 4-amino CGA; **5**, CGA; **6**, cytosine. The fragmentation patterns of the parent ions are shown, and the peaks corresponding to the characteristic fragments are labeled.

conferred the production of gougerotin in *S. coelicolor* M1146. The absence of CMP hydrolase gene in gougerotin cluster indicated that cellular cytosine is directly used for coupling with UDP-glucuronic acid.

The appearance of D-amino acid residues is widespread in natural peptide antibiotics (Martínez-Rodríguez et al., 2010). However, the presence of D-amino acid in peptidyl nucleoside antibiotic is not common. Base on sequence analysis, *gouG*, *gouI*, and *gouL* are suggested to be responsible for the de novo biosynthesis of serine. However, two questions need to be answered before the elucidation of steps involved in serine biosynthesis.

(1) Are these three genes sufficient for the formation of D-serine, or are other genes within or outside the gougerotin cluster involved? In ribosomally synthesized polypeptide antibiotics, the conversion of L-amino acid to D-amino acid results from a two-step reaction involving a lantibiotic synthetase and a dehydrogenase (Cotter et al., 2005), whereas the epimerization domain of nonribosomal peptide synthetases (NRPSs) is responsible for the conversion of L-amino acid to D-amino acid in nonribosomal peptide antibiotics (Li and Jensen, 2008; Stein et al., 2005).

(2) Does the conversion of L-serine to D-serine occur before or after the incorporation into the nucleoside skeleton? The conversion of L-amino acid to D-amino acid occurred after translation in peptide antibiotics (Cotter et al., 2005), whereas the conversion was accomplished along with the extending chain in nonribosomal peptide antibiotics (Li and Jensen, 2008; Stein et al., 2005). Substitution of L-amino acid with D-amino acid in antibiotics can change their physical properties (Molhoek et al., 2011) and antimicrobial activities (Cotter et al., 2005; Kawai et al., 2004). D-serine is presented in the structure of gougerotin, whereas L-serine is found in ningnanmycin. The existence of D-type or L-type serine in gougerotin and ningnanmycin suggests that they may differ in both physical features and biological activities.

Assembly of the nucleoside and peptidyl moieties with peptide bond is one of the unique features of peptidyl nucleoside antibiotics. In the case of gougerotin, the free amino acid substrates are first activated by CoA-binding domain-containing protein (GouK), and the activated seryl or sarcosyl groups were then transferred by acyl-CoA *N*-acyltransferase (GouJ) from acyl-CoA to the amino group of nucleoside moiety. The unsuccessful feeding experiments with sarcosine suggested that sarcosyl-CoA was formed in a two-step mechanism. Glycine was first converted to glycyl-CoA by GouK and subsequently methylated by GouN to generate sarcosyl-CoA (Figure 6). This is different from the other peptidyl nucleoside antibiotics. In the biosynthesis of nikkomycins, polyoxins, and blasticidin S, SanS and its homologs (PolG and BlsI) are suggested to be responsible for the peptide bond formation (Chen et al., 2009; Cone et al., 2003; Li et al., 2011). SanS, PolG, and BlsI belong to the ATP-grasp-fold superfamily of enzymes. Members of this superfamily are characterized by the unique structure of their ATP-binding site (ATP-grasp fold), and they catalyze the ATP-dependent reaction of a carboxylic acid with a nucleophile via the formation of an acylphosphate intermediate (Fawaz et al., 2011). For puromycin, attachment of the tyrosine moiety to the nucleoside skeleton is predicted to be mediated by Pur6, a protein that shows low similarities with peptide synthetases and lacks an

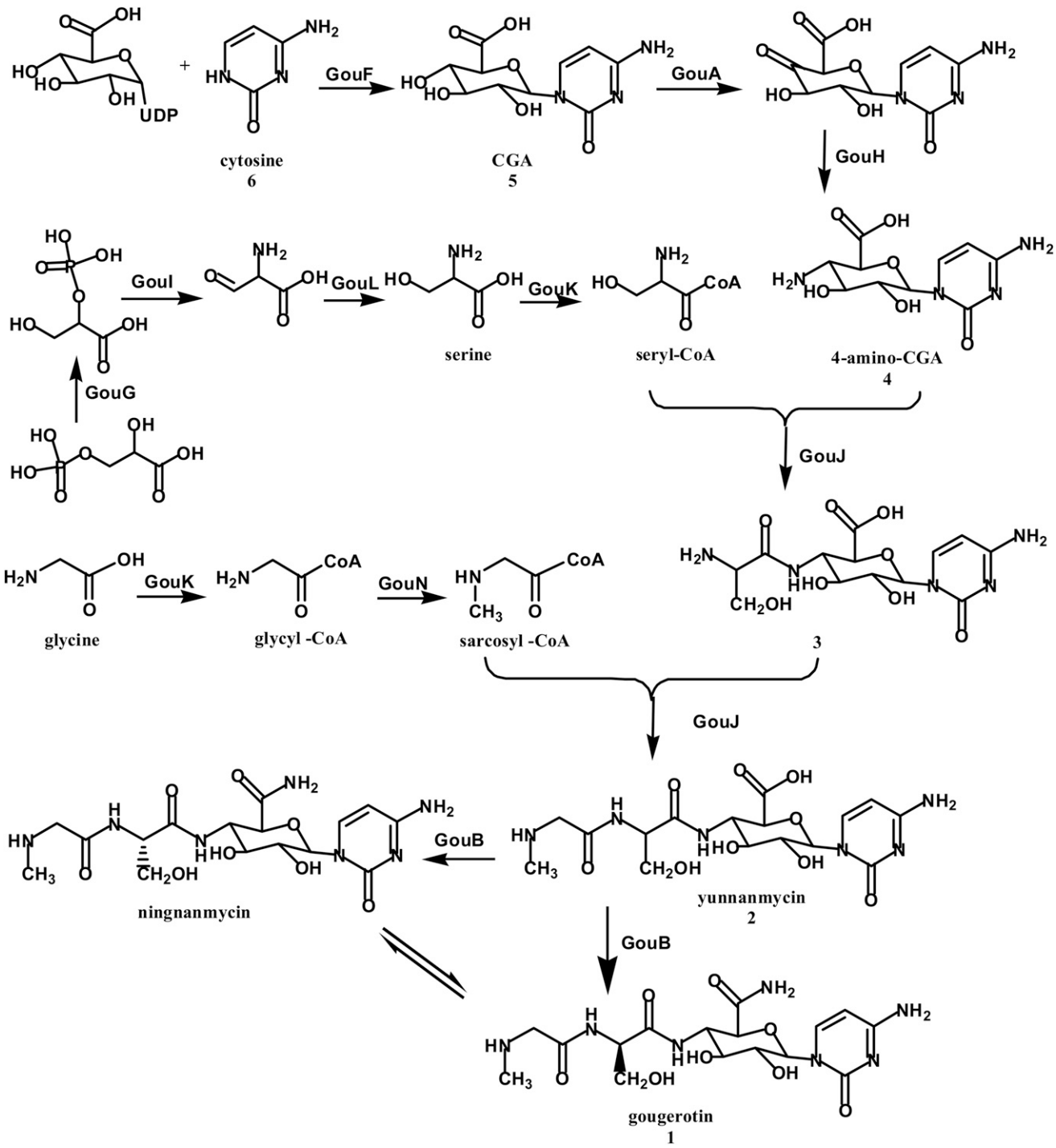

Figure 6. Proposed Biosynthetic Pathway of Gougerotin in *S. graminearus*
The proteins assigned for the proposed catalytic reactions are indicated. The intermediates identified were also labeled numerically.

AMP-binding domain (Tercero et al., 1996). tRNA-dependent peptide bond formation mediated by transferase was also reported in biosynthesis of the pacidamycin group of pentapeptidyl nucleoside antibiotics (Zhang et al., 2011). The mechanisms of peptide bond formation need to be studied further for the elucidation of this group of important antibiotics.

SIGNIFICANCE

Gougerotin, a specific inhibitor of protein synthesis, displays broad biological activities. However, to our knowledge, we knew nothing about the gougerotin biosynthesis prior to when this study was initiated. In this study, we

cloned and characterized the gougerotin biosynthetic gene cluster from *S. graminearus*. Our findings provided important insights into the biosynthesis of gougerotin. The characterization of the biosynthetic pathway indicated that free cytosine was coupled with UDP-glucuronic acid and that the nucleoside skeleton was joined with its two amino acid substrates sequentially. This study opens up venues for future development of compounds with improved properties and rational design of peptidyl nucleoside antibiotics through combinatorial biosynthesis.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, Fosmids, Primers, and Culture Conditions

Bacterial strains, plasmids, and fosmids used in this study are listed in Table S2, and primers are listed in Table S3. *S. graminearus* CGMCC 4.506, *S. coelicolor*, and their derivatives were grown on MS agar or in YEME liquid medium at 28°C. *E. coli* Top10 was used as a general host for propagating plasmids. *E. coli* BW25113 (pKD20) was used for construction of recombinant plasmids via λ-Red-mediated recombination technology. *E. coli* ET12567 (pUZ8002) was used as a host for transferring DNA from *E. coli* into *Streptomyces* by intergeneric conjugation. General approaches for *E. coli* or *Streptomyces* manipulations were performed according to standard methods by Kieser et al. (2000) and Sambrook and Russell (2001). The final antibiotic concentrations used for selection of *E. coli* were as follows: 100 μg/ml ampicillin, 100 μg/ml apramycin, 100 μg/ml kanamycin, 12.5 μg/ml chloramphenicol, and 15 μg/ml tetracycline. The final antibiotic concentrations used for selection of *S. graminearus* were as follows: 25 μg/ml nalidixic acid, 25 μg/ml thiostrepton, and 20 μg/ml erythromycin. The final antibiotic concentrations used for selection of *S. coelicolor* were as follows: 25 μg/ml nalidixic acid and 50 μg/ml apramycin.

DNA Isolation, Manipulation, and Sequencing

DNA isolation and manipulation in *E. coli* and *Streptomyces* were carried out according to standard methods by Kieser et al. (2000) and Sambrook and Russell (2001). PCR amplifications were carried out on a Bio-Rad DNA Engine Peltier Thermal Cycler using either Taq DNA polymerase or KOD plus DNA polymerase. Primer synthesis and DNA sequencing were performed at the Shanghai Invitrogen Biotech and Beijing Institute of Genomics, Chinese Academy of Sciences.

Genomic Library Construction and Screening

The genomic library of *S. graminearus* was constructed in CopyControl pCC1FOS according to the manufacturer's instructions (catalog no. CCFOS110; Epicenter Technologies). Primer pair gouF-F/R was used for screening the fosmid library by PCR. The positive clones were identified in 96-well plates.

Sequence Analysis

The nucleotide sequence reported in this paper is available in the GenBank database under accession number JQ307220. The ORFs were deduced from the sequence with the FramePlot 4.0beta program (http://nocardia.nih.go.jp/fp4). The corresponding deduced proteins were compared with other known proteins in the databases by available BLAST methods (http://www.ncbi.nlm.nih.gov/blast/).

Production, Isolation, and Analysis of Gougerotin

For gougerotin production, spore suspensions were inoculated in liquid YEME and incubated for 48 hr as seed culture. A total of 5 ml seed culture was then transferred into 50 ml fermentation medium (2% glucose, 1% soluble starch, 0.5% yeast extract, 0.5% peptone, 0.3% NaCl, 1% soya power) and incubated with shaking (220 rpm) for an additional 4–5 days at 28°C.

For gougerotin isolation, the culture broth of *S. graminearus* was centrifuged, and the pH of the supernatant was adjusted to 3.0 with oxalic acid. Then it was boiled for 10 min and centrifuged. The supernatant was chromatographed on a Dowexs 50WX2 (H^+, 100–200 mesh; Sigma-Aldrich) column and then it was eluted with 0.3 N ammonia solution. The collected fractions were detected using phosphotungstic acid as indicator, the samples with blue color were further concentrated to a small volume in vacuo (below 40°C), and immediately adjusted pH to 4.5 with acetic acid. Then, 8 vol of 95% ethanol was added. After leaving the mixture at 4°C overnight, the precipitate was collected by centrifugation. The dried powder was subsequently dissolved in water and analyzed by HPLC. Separation of the gougerotin was achieved with Agilent 1100 HPLC system and ZORBAX SB-C18 column (5 μm, 9.2 × 250 mm). HPLC conditions were as follows: 92% H_2O (0.1% TCA) + 8% methanol, flow rate = 1 ml/min, UV = 276 nm. The targeted fraction was collected and lyophilized for further analysis. MS analysis and tandem mass spectrometry analysis were carried out on Triple-Quadrupole LC-MS/MS (Agilent 1260/6460) in positive mode.

For gougerotin analysis, the culture broth was centrifuged, and the supernatant was analyzed by HPLC with Agilent 1100 HPLC system and ZORBAX SB-C18 column (5 μm, 4.6 × 250 mm). HPLC conditions were as follows: 92% H_2O (0.1% TCA) + 8% methanol, flow rate = 1ml/min, UV = 276 nm. The targeted fraction was collected and lyophilized for further MS and MS/MS analyses.

Heterologous Expression of Gougerotin Biosynthetic Cluster

For the heterologous production of gougerotin, pSET152::D6-4H was constructed to determine whether the 28.7 kb DNA fragment in fosmid D6-4H contains all genes essential for gougerotin biosynthesis. Fosmid D6-4H was introduced into *E. coli* BW25113/pKD20 by electroporation. Plasmid pSET152 was linearized with EcoRV and introduced into *E. coli* BW25113/pKD20 containing fosmid D6-4H. Homologous recombination occurred in *lacZα* region and then pCC1FOS backbone of D6-4H was replaced with pSET152 to generate recombinant plasmid pSET152::D6-4H (Figure S4A). The resulting pSET152::D6-4H was respectively introduced into *S. coelicolor* M145 and *S. coelicolor* M1146 by intergeneric conjugation and then the recombinant strains M145-D6-4H and M1146-D6-4H were obtained. The authenticity of these recombinant strains was verified by PCR using gouR-UF/UR, gouH-UF/UR, and orf1-VF/VR primer pairs.

Construction of Mutants

Several mutants were generated for analysis of gougerotin biosynthesis. Gene replacements and gene deletions were constructed in *E. coli* BW25113/pKD20 by using the λ-Red-mediated recombination method. For the construction of *gouH* and *gouL* inactivation mutant in *S. graminearus*, we first constructed pSVgouH-TA and pKC1139EK::D6-4H (see the Supplemental Experimental Procedures). A 6.4 kb DNA fragment containing the thiostrepton/apramycin-resistance cassette and *gouH*-flanking sequences was excised from pSVgouH-TA and used to replace *gouH* in pKC1139::D6-4H. A 2.4 kb DNA fragment was amplified using primers gouL-DF/gouL-DR with pSV152TA as template. The amplified fragment containing the thiostrepton/apramycin-resistance cassette and *gouL*-flanking sequences was used to replace *gouL* in pKC1139::D6-4H. After restriction digestion analysis and PCR verification, the mutant cluster was electroporated into *E. coli* ET12567/pUZ8002 and transferred into *S. graminearus* by intergeneric conjugation. Spores of transformants were harvested, series diluted, and spread on MS agar containing thiostrepton. After growing for 4 days at 37°C, colonies were replicated on MS agar plates containing thiostrepton or erythromycin. Double-crossover exconjugants are erythromycin sensitive (*Erm*^{s}) and thiostrepton resistant (*Tsr*^{r}). *Erm*^{s} strains are then verified by PCR and Southern blot analysis.

For the construction of *gouH* in-frame deletion mutant in *S. coelicolor*, the 6.4 kb DNA fragment was used to generate pfosH-TA with the inactivation of *gouH* in fosmid D6-4H. The thiostrepton/apramycin-resistance cassette is flanked by FRT (Flippase recognition target) sites, which allows for excision of the antibiotic cassette following expression of the FLP-recombinase (Flippase recombination enzyme) in *E. coli* BT340. The pCC1FOS backbone of the mutant clusters was then replaced with pSET152 to generate pSET152::gouH-IFD.

To construct *gouF* and *gouK* in-frame deletion mutants in *S. coelicolor*, the thiostrepton/apramycin- resistance cassette was amplified from pSV152TA with gouF-DF/DR and gouK-DF/DR, respectively. The resulting cassettes (F-*tsr*/*apr*^{R} and K-*tsr*/*apr*^{R}) were used to generate pfosF-TA and pfosK-TA after the inactivation of *gouF* and *gouK* in fosmid D6-4H. pSET152::gouF-IFD and

pSET152::gouK-IFD were constructed from pfosF-TA and pfosK-TA using a similar procedure of pSET152::gouH-IFD. After restriction digestion analysis and PCR verification, pSET152::gouF-IFD, pSET152::gouH-IFD, and pSET152::gouK-IFD were transferred into *S. coelicolor* M1146, and the recombinant strains gouF-IFD, gouH-IFD, and gouK-IFD were obtained.

Complementation of *gouL* Mutant

For complementation experiments, the constitutive *hrdB* promoter was used to drive the expression of *gouL*. The *hrdB* promoter was amplified from *S. graminearus* genomic DNA with primers hrdB-PF/hrdB-PR. The coding region of *gouL* was amplified from fosmid D6-4H with primer pair gouL-CF/gouL-CR. The *hrdB* promoter was cut with XbaI, and the *gouL*-coding region was cut with EcoRI. Both the *hrdB* promoter and *gouL*-coding fragment were ligated together with XbaI/EcoRI double-digested pSET152Erm. The resulting pSET152::gouLc was introduced into the *gouL* mutant to obtain the complementation strain gouLc.

Determination of the Minimal Gougerotin Biosynthetic Gene Cluster

In order to determine the boundaries of gougerotin biosynthetic gene cluster, a series of truncation constructs (pD64H-R, pD64H-1, and pD64H-N) were generated (described in detail in Figures S4B and S4C and the Supplemental Experimental Procedures). The resulting constructs were verified by restriction digestion and then transferred into *S. coelicolor* M1146 to generate BDorf(−1)–(−6), BDgouN and BDorf1–4 strains. The boundaries of gougerotin gene cluster were determined by monitoring gougerotin production with HPLC analysis.

ACCESSION NUMBERS

The GenBank accession number for the 28.7 kb insert of fosmid D6-4H reported in this paper is JQ307220.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2012.10.017.

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